Immobilization of laccase from *Trametes hirsutus* on allumina and decolurization of textile dye for color removal of effluent.

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1. Introduction

Laccase (EC 1.10.3.2) is a multicopper oxidase which reduces oxygen to water and simultaneously performs one-electron oxidation of many aromatic substrates [7]. Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols , ortho- and para-diphenols, but it be able also to oxidize other substrates such aromatic amines, syringaldazine, and non-phenolic compounds to form free radicals [8].
[6]

2. Materials and methods

2.1 Instruments

2.2 Covalent Immobilization

Alumina (Al_2O_3) tablets pellets (7 mm) and sferical pellets (3 mm) were silinized, immerse in γ - aminopropyltriethoxy silane 2.5% (V/V) [1] in solution of acetone at 45ºC for 24 h.
The pellets were washed whit distilled water and immersed in 2% (V/V) of aqueous glutaraldehyde solution [2] for 2 h at room temperature. Re-washed whit distilled water, therafter the support was dry at 60ºC for 1h.
At 5 g of pellets was add in 10 ml of the crude enzyme preparation, in 0.1 M sodium acetate (pH 5), for 5 h at room temperature.
Immobilized protein was determined by protein analysis according to the method of Bradford [3] by using bovine serum albumin for the calibration.
The immobilized enzyme pellets were washed whit phosfate buffer solution of pH 7.0 and kept a refrigerated until further use.

2.3 Enzyme activity

A Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) from *Trametes hirsutus* were supplied by VTT Biotechnology (Espoo, Finland).
Initially for the immobilezed laccase it was used the Jong method [4] but its three steps were laborius. It was not possible used the best alternative that is a direct measurement of the oxygen consumption. Finaly it was used the ABTS test [5] based on a direct one electron oxidation of substrate that is still one of the best indirect tests for determining the activity of oxidative enzymes.

2.3.1 Method Jong

Laccase activity was determined using 2.6-dimethoxyphenol (DMP) as a substrate [4]. The reaction mixture contained 50 mmol sodium malonate (pH 4.5), 1mmol DMP, 1mmol $MnSO_4$, and 700 μl sample in a total volume of 1ml.
The formation of an orange/brownish dimer was followed spectrophotometrically at 468 nm. Activities for both these enzymes were calculated using the molecular extinction coefficent of 49.6 $mM^{-1}cm^{-1}$ and expressed as IU wich is defined as one $\mu mol.min^{-1}$ of substrate converted.

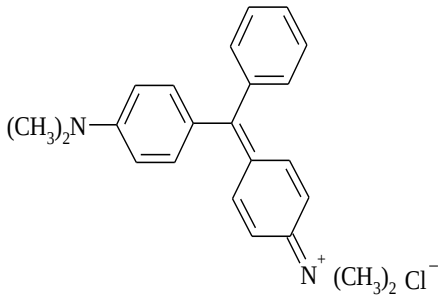
2.3.2 Method ABTS

Laccase activity was determined by using ABTS [2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] as a substrate [5]. The reaction mixture

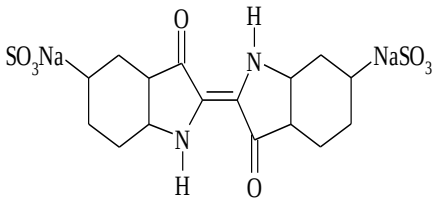
contained 0.5 mmol ABTS, and 1 ml sample, diluited in 0.1 M sodium acetate (pH 5), in a total volume of 2 ml.
Oxidation of ABTS was followed spectrophotometrically at 420 nm. Activities for both these enzymes were calculated using the molecular extinction coefficent of $3.6*10^4$ $mM^{-1}cm^{-1}$ and expressed as IU wich is defined as one $\mu mol.min^{-1}$ of substrate converted.

2.4 Dyes

Two model dyes were used to study their influence in the pH profile activity of Laccase. A cationic dye, crystal violet (basic violet 3, C.I. 42555) and an anionic dye, indigo carmine (acid blue 74, C.I. 73015). The dyes were purchased from Sigma with 90% of purity. The chemical structure of these two dyes is represented in Figure 1.



Basic Violet 3



Acid Blue 74

Figure 1: Chemical structure of the two dyes used in this study.

2.5 Decolourisation

Dye solutions in buffer (95 mL) were incubated with 100 μL of enzyme solution in a shaker bath operating at 90 rpm. Samples of the reaction mixture were taken at different periods of time to measure the dye absorbance and the percentage of decolourisation was calculated. In the temperature activity profile experiments, the enzymatic reaction was performed at pH 5.0. The activity-pH profile of laccase enzyme was studied in the range of pH 3-9 and in these experiments the temperature was set at 50 ºC. The buffer systems used in these experiments are listed in Table 1 and were prepared at 0.1 *M* concentration. Control tests were also performed in order to observe the effect of buffer and temperature alone on dye decolourisation and this effect was subtracted to the activity observed in the enzymatic reactions.

pH	Buffer system
3	Tartaric acid-NaOH
4	Acetic acid-NaOH
5	Acetic acid-NaOH
6	Phosphoric acid-NaOH
7	Phosphoric acid-NaOH
8	Tris-HCl
9	Tris-HCl

Table 1: Buffer systems used in activity-pH profile experiments.

3 Results and Discussion

3.1 Differences of allumina supports

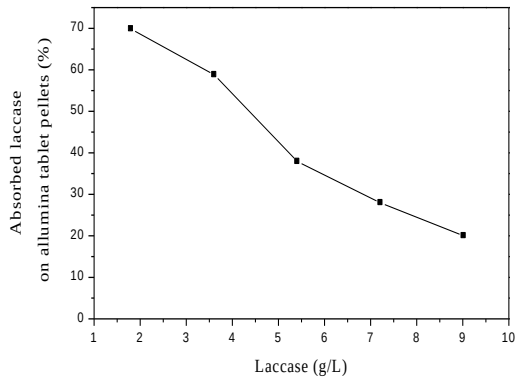


Figure 1:

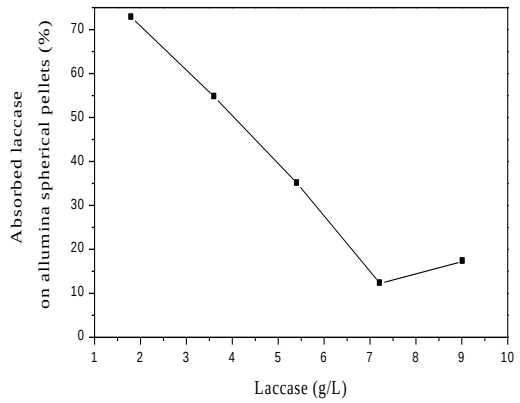


Figure 2:

3.2 Effect of temperature on enzyme activity

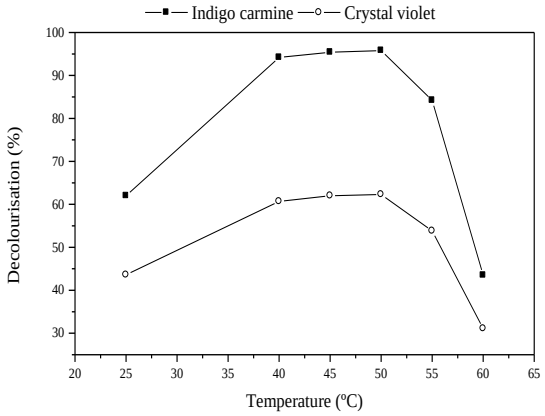


Figure 3: Activity-temperature profile of *Trametes hirsutus* laccase for the two dyes (activity measured as percentage of decolourisation after 24 hours incubation, pH 5.0).

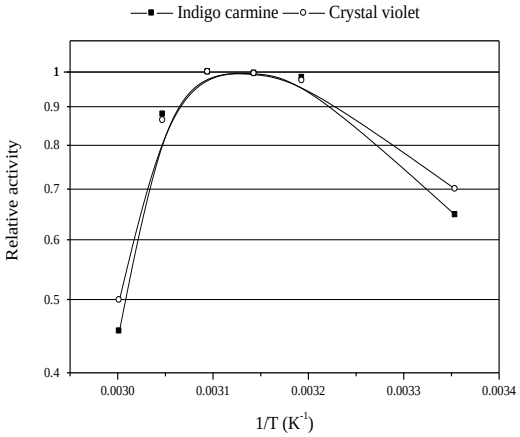


Figure 4: Arrhenius plot for the activity of *Trametes hirsutus* laccase in indigo carmine and crystal violet dyes (pH 5.0, 24 hours).

3.3 Effect of pH on enzyme activity

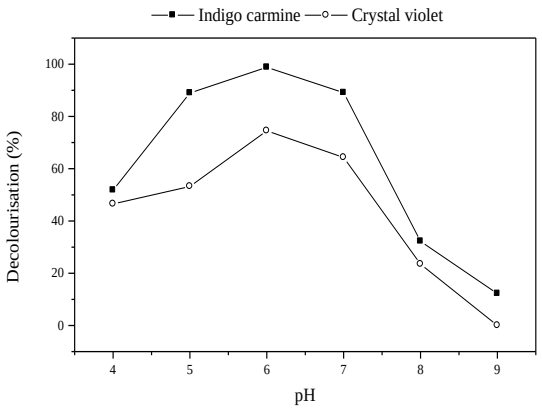


Figure 5: Activity-pH profile of *Trametes hirsutus* laccase for the two dyes (measured as percentage of decoulorisation after 24 hours incubation, 50 ^{o}C).

4 Further direction for research

4.1 Reactors

4.1.1 Recyrculed reactor

Immobilized laccase will perform in a thermostated packed bed reactor column, 2.5 cm × 20 cm, obtained from Sigma. The dye solution will pump into a column with a peristaltic pump. The immobilized laccase will applied in the reactor to study the stability and the half-life time of the enzyme for the degradation of dye solution.

4.1.2 Open Reactor

For immobilized laccase residence time optimization, in the dye solution, was created a open reactor system. The fixed allumina support was

4.2 Activity of immobilized laccase

5 Conclusion

References

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